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SLOWLY INACTIVATING POTASSIUM CURRENT IN CULTURED BULL-FROG PRIMARY AFFERENT AND SYMPATHETIC NEURONES

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SUMMARY

1. Cultured bull-frog dorsal root ganglion cells were voltage clamped in the whole-cell configuration. The cells were superfused with a nominally calcium-free Ringer solution containing tetrodotoxin (3 μM), magnesium (10 mM), cobalt (1 mM), barium (2 mM), 4-aminopyridine (3 mM) and caesium (2 mM).

2. Step depolarizations (10–40 mV, 100–300 ms) from a holding potential close to the rest (typically −70 mV) evoked an outward current (I_K) followed by an outward tail current. The peak amplitude of the current was reduced to less than 10% by tetraethylammonium (30 mM).

3. I_K developed to its peak in 200 ms at −30 mV. Tail currents reversed at potentials that changed according to the logarithm of the extracellular potassium concentrations.

4. Tail currents declined to the baseline according to an exponential function of time ($\tau \simeq 40$ ms at −60 mV) and its reciprocal time constant increased e-fold with a 13 mV hyperpolarization.

5. The current inactivated during sustained (1–20 s) depolarizing pulses according to a single exponential function ($\tau \simeq 3$ s).

6. The peak amplitude of I_K at −30 mV was progressively increased as the holding potential was made more negative than −70 mV reaching the maximum with step depolarizations from −120 mV. Reversed phenomenon was observed as the holding potential was made less negative than −70 mV.

7. The removal of the steady-state inactivation occurred along with a single exponential function and the time constant was decreased from 70 ms at −70 mV to 10 ms at −120 mV.

8. It is suggested that a slowly inactivating potassium current which we called I_K in amphibian sensory neurones could be a class of a 'delayed' rectifier potassium current. A potassium current with properties indistinguishable from those which have been described for the sensory I_K also occurred in cultured bull-frog sympathetic neurones.

9. Forskolin (1–30 μM) and 1,9-dideoxy forskolin (10 μM) reduced the amplitude of I_K by up to 85% but these actions were not mimicked by any of 8-bromo-cyclic AMP (1 mM), dibutyryl cyclic AMP (1 mM) and 3-isobutyl-1-methylxanthine (1 mM). A

hydrophilic forskolin analogue, 7-*O*-hemisuccinyl-7-deacetyl forskolin (10 μM), was about one-tenth as potent as forskolin (10 μM).

INTRODUCTION

In general, the two main classes of voltage-dependent, calcium-insensitive potassium currents that have often been distinguished are the 'delayed' (Hodgkin & Huxley, 1952) and the 'transient' (Conner & Stevens, 1971) although the kinetic scheme for both currents as well as their sensitivity to tetraethylammonium (TEA) and 4-aminopyridine (4-AP) varies from one preparation to the other (see Dubois, 1983; Hille, 1984; Rudy, 1988).

Because of this diversity, the distinction between the two currents cannot be clearly made in many vertebrate nerve cells and there appear to be intermediate classes (see Rudy, 1988). For example, the 'transient' current in some mammalian sensory neurones (Kasai, Kameyama, Yamaguchi & Fukuda, 1986; Stansfeld, Marsh, Halliwell & Brown, 1986) inactivates as slowly as the 'delayed' current does in squid giant axon (Ehrenstein & Gilbert, 1966) and amphibian sensory and sympathetic neurones (Kostyuk, Veselovsky, Fedulova & Tsyndrenko, 1981; Adams, Brown & Constanti, 1982).

During the course of experiments in which an attempt was made to characterize a non-inactivating potassium current (M-current; Brown & Adams, 1980) of bull-frog primary afferent neurones (Tokimasa & Akasu, 1990*b*), we have noticed that the activating M-current is superimposed by an additional, slowly inactivating outward current even in the presence of 4-AP (1–2 mM). The current was still present under conditions at which there are no M-current and calcium-activated potassium currents.

This outward current might be a subclass of the 'delayed' current since it was hardly detected in a TEA-rich (30–90 mM) solution (Tokimasa & Akasu, 1990*b*, *c*) although a slow inactivation may also occur for the 'transient' current (see above) and the sensitivity of the sensory 'transient' current to 4-AP also varies among preparations (Ito & Maeno, 1986; Kasai *et al.* 1986; Stansfeld *et al.* 1986; Mayer & Sugiyama, 1988). Alternatively, the outward current might be the 'transient' current since the threshold voltage for the current activation was at least 20–30 mV more negative than that which has been reported for the sensory 'delayed' current in frog (~ -25 mV, Ito & Maeno, 1986) and rat (~ -35 mV, Kostyuk *et al.* 1981; Mayer & Sugiyama, 1988).

The main purpose of the present study was to characterize this as yet unidentified slowly inactivating outward current in dissociated bull-frog dorsal root ganglion cells. Because of a relatively low threshold for the current activation, it was particularly asked whether this outward current could contribute to the steady-state membrane conductance. Bull-frog sympathetic neurones were also studied since the kinetic scheme has been established for the 'delayed' current and the current also shows a slow inactivation (Adams *et al.* 1982). A preliminary account of portions of the present study has appeared in abstract form (Tsurusaki, Tokimasa & Akasu, 1990).

METHODS

All the experiments in the present study were carried out at 22–24 °C. Statistics are expressed as means ± S.E.M. for the cells tested.

Tissue culture

Small (200–250 g) bull-frogs (*Rana catesbeiana*) were used. After decapitation and pithing, dorsal root ganglia were rapidly removed and placed in Ringer solution of the following composition (mM): NaCl, 112; KCl, 2; $CaCl_2$, 1·8; HEPES, 4 and Tris, 1 (pH adjusted with HCl/NaOH to 7·2). Cultured neurones were prepared as described previously (Tokimasa & Akasu, 1990*b*).

Electrophysiological experiments

Techniques for the whole-cell recordings were essentially the same as those which have been described previously (Tokimasa & Akasu, 1990*b*). In brief, a sample-and-hold/voltage-clamp amplifier (Axoclamp 2A) was used at the switching frequency of 10–12 kHz. The head stage current gain of the amplifier was 0·1. This indicated that clamping currents as large as ±20 nA could be applied to the cells. Pipettes for the patch clamp had tip resistances of 2–6 MΩ when filled with a solution having the following composition (mM): KCl, 100; EGTA, 1; $MgCl_2$, 4; adenosine 5′-triphosphate (disodium salt), 5; HEPES (sodium salt), 2·5 (pH adjusted with ~ 6 mM-KOH to 7·0) (see Jones, 1989). The equilibrium potential for potassium ions, calculated from the internal and external solutions, was −100·0, −59·5 and −42·0 mV with the concentration of potassium ions in the superfusate at 2, 10 and 20 mM, respectively. These values are compatible with those which have been reported previously (Jones, 1989).

Isolation of I_K from other membrane currents

Voltage dependence of a slowly inactivating delayed rectifier potassium current which we called I_K was studied in a nominally calcium-free solution containing tetrodotoxin (TTX; 3 μM), magnesium (10 mM), barium (2 mM), caesium (2 mM), 4-AP (3 mM) and cobalt (1 mM). TTX was used to eliminate an inward sodium current (Ishizuka, Hattori & Akaike, 1984). Calcium was omitted from and magnesium and cobalt were added to the Ringer solution to eliminate an inward calcium current (Ishizuka *et al.* 1984; Fox, Nowycky & Tsien, 1987; Bean, 1989) and subsequently activated potassium current and chloride current (Inoue, Sadoshima & Akaike, 1987; Morita & Katayama, 1989). Barium was used to eliminate the M-current (Tokimasa & Akasu, 1990*b*). Caesium was used to eliminate the H-current (Tokimasa & Akasu, 1990*a*, *b*). 4-AP was used to reduce the amplitude of the A-current (Ito & Maeno, 1986).

A-current

The time course with which the A-current inactivates was re-examined in the present study. For this purpose, the cells were superfused with a nominally calcium-free Ringer solution containing TTX (3 μM), magnesium (10 mM), tetraethylammonium (TEA; 30 mM) and caesium (2 mM). In order to avoid a depolarizing shift in the steady-state activation and inactivation curves of the A-current, cobalt was not used to facilitate the suppression of the inward calcium current (Mayer & Sugiyama, 1988). For the same reason, barium was not used to eliminate the M-current and therefore a fraction (≃ 65%) of the M-current remained in this nominally calcium-free solution (see Tokimasa & Akasu, 1990*b*, *c*).

H-current

The H-current was recorded in a nominally calcium-free and potassium-rich (5 mM) Ringer solution containing TTX (3 μM), cobalt (2 mM), magnesium (10 mM), barium (2 mM), TEA (20 mM) and 4-AP (2 mM) (see Tokimasa & Akasu, 1990*b*). The concentration of NaCl was reduced from 112 to 80 mM.

Experiments in sympathetic neurones

Some experiments were also carried out on cultured bull-frog sympathetic B-neurones using the methods described previously (Tokimasa & Akasu, 1990*a*, *c*).

Drugs

Drugs used were apamin, β-bungarotoxin, 4-aminopyridine, adenosine 5′-triphosphate (disodium salt), forskolin, adenosine 3′,5′-cyclic monophosphate (sodium salt), 8-bromoadenosine 3′,5′-cyclic monophosphate (sodium salt), N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (sodium salt), phorbol 12-myristate 13-acetate (all from Sigma), 1,9-dideoxy forskolin (Calbiochem), 7-*O*-hemisuccinyl-7-deacetyl forskolin (Calbiochem), protein kinase inhibitor H-8 (Seikagaku Kogyo, Japan), 3-isobutyl-1-methylxanthine (Aldrich), tetraethylammonium chloride (Tokyo Kasei, Japan) and tetrodotoxin (Sankyo, Japan). Phorbol 12-myristate 13-acetate (PMA) was dissolved with dimethyl sulphoxide (3·3 mg ml^{-1}) (Wako Pure Chemicals, Japan) and stored at −20 °C.

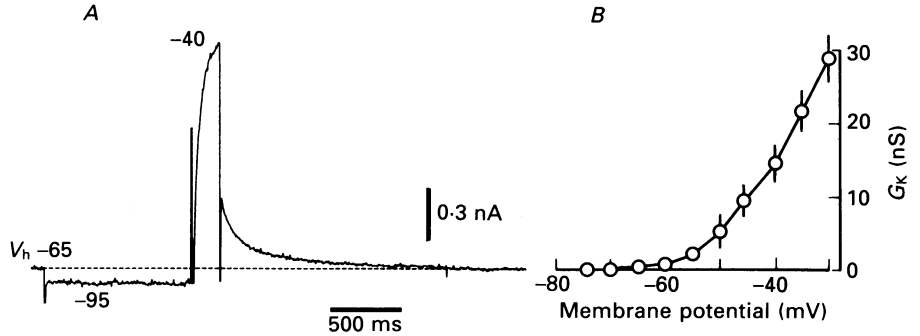

Fig. 1. Delayed rectifier potassium current. *A*, the cell membrane potential was clamped at the holding potential (V_h) of −65 mV and commanded first to −95 mV for 1 s and then to −40 mV for 200 ms. The activated I_K was followed by an outward tail current upon stepping back from −40 to −65 mV. G_K at −40 mV was estimated using an equation of the form $I/(V_h-E)$, where I and E denote the peak amplitude and the reversal potential of the tail current: 415 pA and −88 mV in this cell and hence G_K was thus estimated as 18 nS (see Fig. 2*A* for the determination of the E in this cell). Results obtained from experiments of this type are summarized in *B*. Circles and associated bars denote mean $G_K \pm$ S.E.M. for fifteen cells tested. The cells in this and following figures were superfused with a nominally calcium-free Ringer solution containing 4-AP (3 mM) and barium (2 mM) (see Methods for its composition).

RESULTS

Delayed potassium current in primary afferent neurones

Results described were obtained from large spheroidal A-cells having a mean diameter greater than 45 μm ($n = 90$) (see Tokimasa & Akasu, 1990*b*). Unless otherwise mentioned, the cells were superfused with a nominally calcium-free Ringer solution (see Methods). The usual experimental protocol adopted for studying a 'delayed' potassium current is illustrated in Fig. 1: the potassium current is referred to as I_K in the present account. The cell membrane potential was held near the resting potential ($V_h = -65$ mV) and commanded to a hyperpolarized level ($V_1 = -95$ mV) for 1 s before stepping to a depolarized level ($V_2 = -40$ mV) for 200 ms. Activated I_K during the second pulses was followed by an outward tail current upon stepping back to the holding potential of −65 mV (Fig. 1*A*).

The peak amplitude of the tail current (I) was measured to estimate the potassium conductance at given potentials (G_K (V_2)) using an equation of the form, $G_K(V_2) = I/(V_h-E)$, where E denotes the reversal potential of I_K (see below). In fifteen cells with

V_2 at -30 mV, G_K (-30) was thus estimated at 29 ± 2 nS (Fig. 1*B*). $G_K(V_2)$ fell off to zero between -65 and -70 mV indicating the threshold for the I_K activation (Fig. 1*B*).

Reversal potential of tail currents

For the determination of the reversal potential of I_K, the second in a paired pulse was followed by a third pulse (V_3) for commanding the cell membrane potential

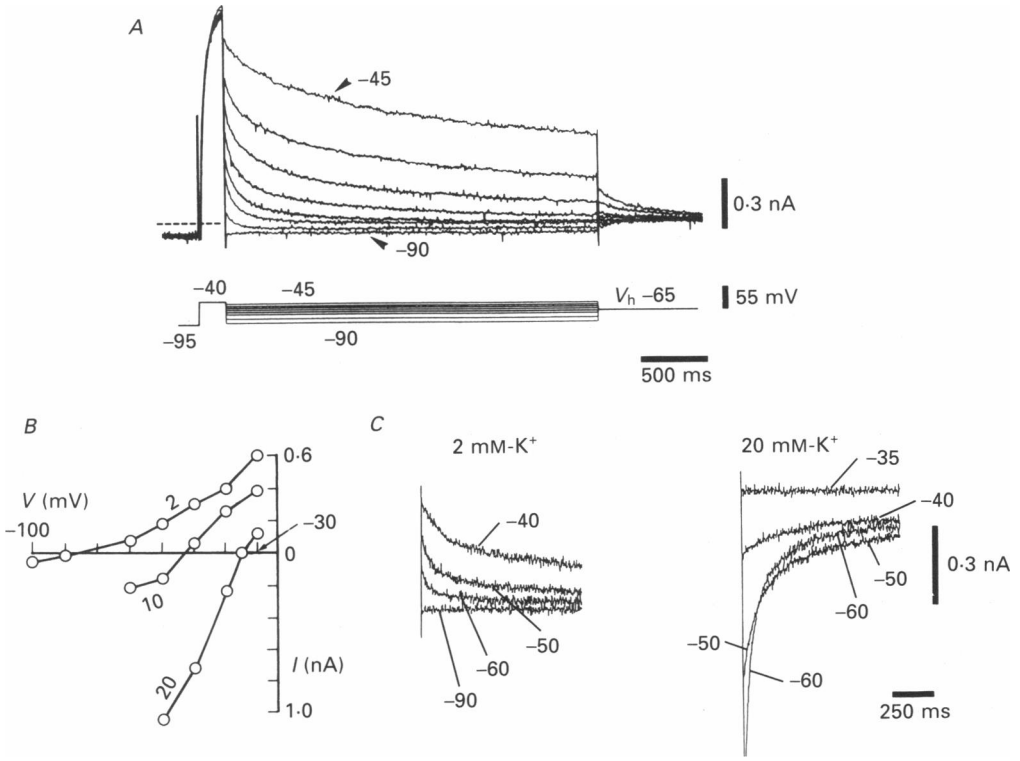

Fig. 2. Potassium dependence of the I_K reversal potential. *A*, results were obtained from the same cell as that in Fig. 1*A* and the same twin-pulse protocol as that in Fig. 1*A* was used to activate I_K. Immediately after the second in the paired pulse, the cell membrane potential was commanded for 2·8 s to various potentials between -45 and -90 mV before stepping back to -65 mV (5 mV increments between -45 and -60 mV and 10 mV increments between -60 and -90 mV). The tail current reversed its polarity from outward to inward at -88 mV indicating the reversal potential of I_K. *B* and *C*, results were obtained from another cell. The cell membrane potential was clamped at the holding potential (V_h) of -43 mV and commanded first to -92 mV for 500 ms. The cell was then depolarized to -30 mV for 500 ms and finally polarized between -35 and -100 mV before stepping back to -43 mV. *B*, the peak amplitude of the I_K tail current was plotted in ordinate (in nA, upward for the outward tails) as a function of polarizing levels (abscissa in mV). The reversal potential of the tail current was -86, -52 and -35 mV when $[K^+]_o$ in the superfusate was 2, 10 and 20 mM (indicated beside each curve), respectively. *C*, sample recordings plotted in *B* for $[K^+]_o = 2$ mM (left) and 20 mM (right). The polarizing level is indicated beside each current trace. Note that the peak amplitude of the inward tail current at -60 mV with $[K^+]_o$ at 20 mM is off scale.

between −35 and −115 mV (e.g. between −45 and −90 mV in Fig. 2*A*), The reversal potential was given as a membrane potential at which the amplitude of the tail current was decreased to zero. With the concentration of potassium ions in the superfusate ($[K^+]_o$) at 2 mM, I_K reversed its polarity from outward to inward at −87·4±1·8 mV ($n = 11$, Fig. 2*A* and *B*). The reversal potential was shifted to −48·6±3·5 mV ($n = 3$) and −33·7±1·3 mV ($n = 4$) when $[K^+]_o$ was elevated from 2 to 10 and 20 mM, respectively (Fig. 2*B* and *C*). These values were comparable to those which have been calculated as the equilibrium potential for potassium ions (see Methods), and those which have been reported for the reversal potential of the M-current (Tokimasa & Akasu, 1990*c*). As described in detail further below, it was noticed that the time course with which the tail currents declined to the baseline became faster with membrane hyperpolarizations (Fig. 2*C*).

The reversal potential of I_K was not significantly changed in a nominally sodium-free solution ($n = 3$; NaCl substituted with choline chloride) and in a chloride-deficient (50%) solution ($n = 3$; NaCl substituted with sodium isethionate).

Kinetics of onset

I_K activated along the sigmoidal time course (Fig. 3) normally associated with the classical delayed rectifier current (Hodgkin & Huxley, 1952). This implied that I_K may follow the normal Hodgkin–Huxley kinetic scheme and hence that I_K develops with time as

$$I_K(t) = \bar{I}_K[1-\exp(-t/\tau)]^\gamma. \quad (1)$$

Figure 3*A* shows a plot of the logarithmic transformation of eqn (1):

$$\ln[1-(I_K(t)/\bar{I}_K{}^{\gamma^{-1}}] = -t/\tau, \quad (2)$$

taking $\gamma = 1$, 2 and 3. The plot was clearly non-linear at $\gamma = 1$, and becomes linear at $\gamma = 2$ or 3. Taking γ (minimum) = 2, the time constant for the on currents derived from such plots shortened with depolarization from 93±7 ms at −55 mV ($n = 3$) to 39±5 ms at −30 mV ($n = 4$) (Fig. 3*B*). The reciprocal time constant (τ^{-1}, s^{-1}) increased e-fold with a 30 mV depolarization (Fig. 3*B*).

Kinetics during sustained depolarizing pulses

In all eighty-six cells tested, the amplitude of I_K was decreased during sustained (1–20 s) depolarizations (Fig. 4*A*). The time course with which I_K inactivates at given potentials was measured using a hyperpolarization-induced extra I_K defined as a component to I_K which can be recruited by subjecting the cells to brief (0·5–1 s) hyperpolarizing pre-pulses. An example of this extra I_K at three different potentials is shown as a family of current traces in the lower left panel of Fig. 4*B*. The amplitude of the extra I_K declined single exponentially with time and finally reached a non-zero steady-state value (see Dubois, 1983). In Fig. 4*B*, for example, the extrapolation of a single exponential phase of the extra I_K at −35 mV approached an asymptote at about 25% of its peak amplitude (see further below for this non-zero steady-state I_K). The time constant varied among twelve cells (range, 1·5–3·5 s) but, in a given cell, the time constant was rather independent of the membrane potential between −55 and −25 mV.

The reversal potential of I_K tail current, measured 0·5 and 5·5 s after the onset of step depolarizations to −30 mV, was −89·1±2·2 and −86·4±2·1 mV, respectively,

in three cells (Fig. 4*C*). This implied that the slow decline of I_K amplitude was not due primarily to a depolarizing shift in the reversal potential arising from the accumulation of potassium ions at the perineuronal space.

A membrane current in response to step hyperpolarizations (40–50 mV, 0·5–1 s) from the holding potential of −60 mV changed its polarity from inward to outward

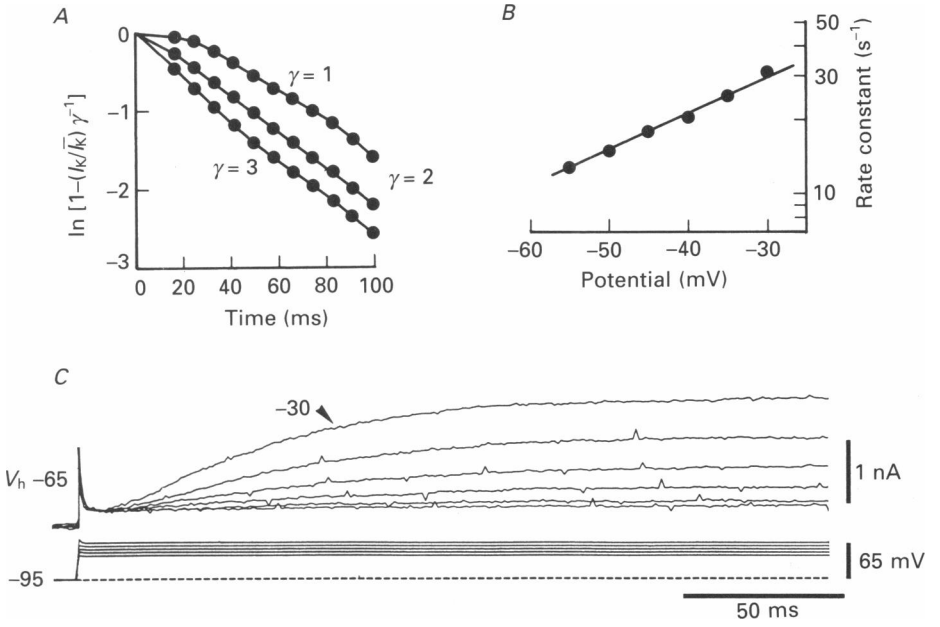

Fig. 3. Sigmoidal I_K onset. Results were obtained from a single cell. The cell was hyperpolarized from the holding potential (V_h) of −65 to −95 mV for 1 s and then subjected to depolarizing step pulses ranging from −60 to −25 mV in 5 mV increments. *A*, ordinate denotes eqn (2) taking $\gamma = 1$, 2 and 3. Abscissa denotes time after the onset of a step depolarization from −95 to −30 mV. *B*, the rate constant (τ^{-1} in s^{-1}) for the activating I_K between −55 and −30 mV assuming that $\gamma = 2$. The slope factor was e-fold/30 mV. *C*, sample recordings plotted in *A* and *B*. I_K at −30 mV is indicated by an arrow-head.

when the $[K^+]_o$ in the superfusate was elevated from 2 to 20 mM in eight of ten cells (Fig. 4*D*). These observations implied that a tonic inward flow of I_K occurs at −60 mV and this inward current was deactivated during the hyperpolarizations.

Kinetics at offset of depolarizing pulses

In contrast to I_K onset, deactivating I_K tail current showed a simple exponential decay with time (Fig. 5). Measurements at different repolarizing potentials showed a clear acceleration with increasingly negative repolarizing potentials. In five cells with $[K^+]_o$ in the superfusate at 2 mM, the mean value of the reciprocal time constant (τ^{-1} in s^{-1}) increased e-fold with a 12·6 mV hyperpolarization (Fig. 5*A* and *B*). The reciprocal time constant between −80 and −100 mV was measured in a potassium-rich (20 mM) solution by taking advantage of an increased driving force for potassium ions (Fig. 5*C* and *D*). In four cells, the mean value for the τ^{-1} (s^{-1}) increased e-fold with a 13·1 mV hyperpolarization. In all nine cells tested, the

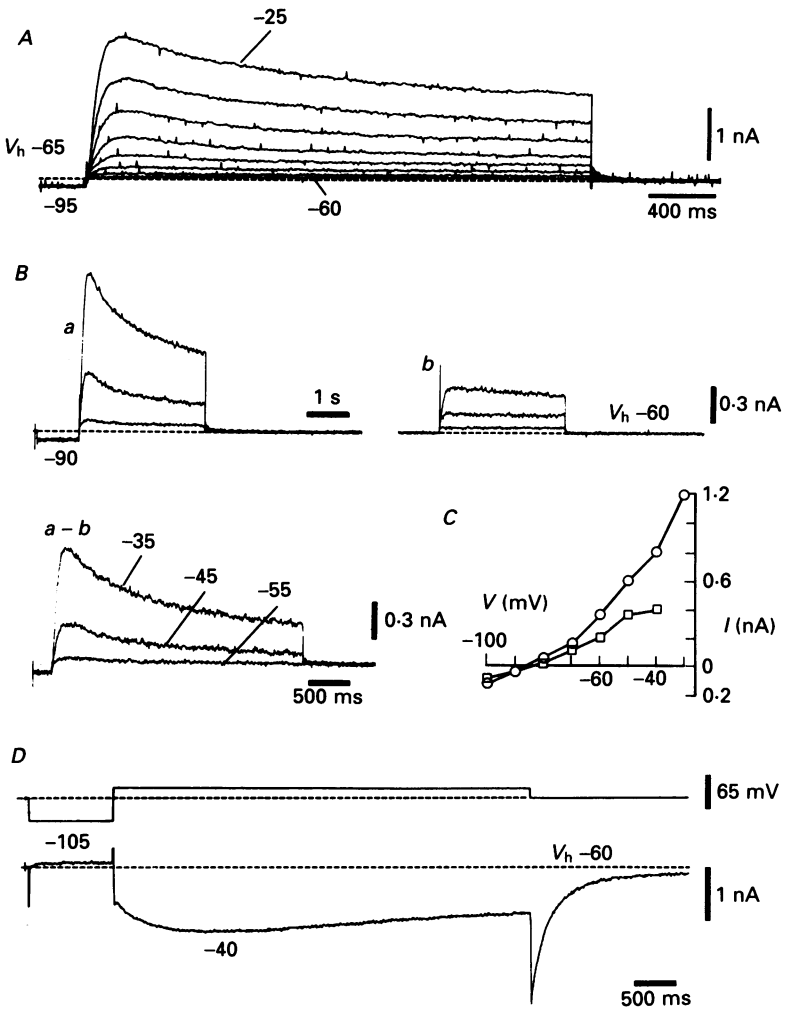

Fig. 4. Inactivation of I_K. Results in *A–D* were obtained from four different cells. *A*, the cell was held at the holding potential (V_h) of −65 mV and subjected first to step hyperpolarizations to −95 mV for 1 s and then to depolarizing step pulses for 3 s ranging from −60 to −25 mV with 5 mV increments before stepping back to −65 mV. *B*, the cell was depolarized for 3 s from a V_h of −60 mV to three different potentials (10 mV increments started from −55 mV) with and without applying hyperpolarizing pre-pulses to −90 mV for 1 s. The trace in the upper left panel (*a*) and that in the upper right (*b*) represent current responses obtained from these experiments. The lower left panel represents the extra I_K recruited by 30 mM hyperpolarizing pre-pulses at −55, −45 and −35 mV (*a*−*b*). *C*, the results were obtained from the same cell as that in Fig. 2*B* and *C* using the same triple-pulse protocol: the duration of the second in the triple-pulse was either 0·5 s (○) or 5·5 s (□). The reversal potential of I_K tail current shifted from −86 to −82 mV by increasing the duration of the second pulse by 5 s. *D*, experiments with $[K^+]_o$ in the superfusate at 20 mM and V_h at −60 mV. The cell was hyperpolarized from −60 to −105 mV for 1 s and then depolarized to −40 mV for 5 s before stepping back to V_h −60 mV. The activated I_K (now inward) showed a slow inactivation resulting in an outward relaxation on the current trace followed by an inward tail current upon stepping back to −60 mV.

τ^{-1} (s^{-1}) was constant at one voltage irrespective of the polarity of the activated I_K and the polarity of the deactivating I_K tail current. Figure 5*E* shows such an example in which the time course for the deactivating I_K tail was tested at the holding potential of −60 mV: the polarity of the activated I_K was outward at −30 mV and inward at −50 mV and the polarity of all the three tail currents was inward.

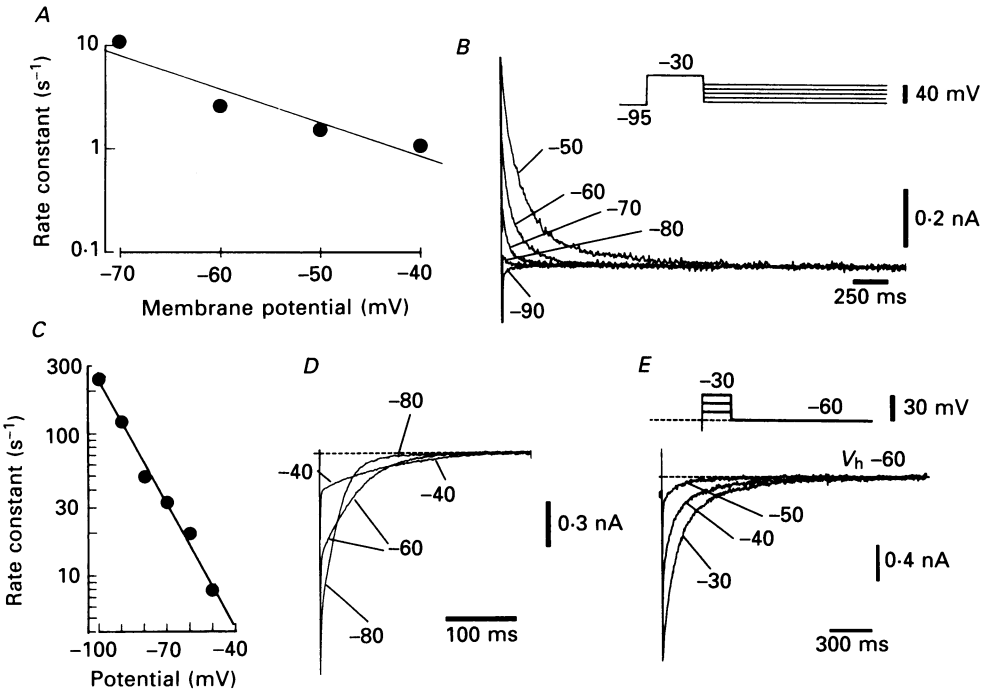

Fig. 5. Deactivating time course of I_K. Results were obtained with the $[K^+]_o$ in the superfusate at 2 mM (*A*, *B*), 20 mM (*C*, *D*) and 10 mM (*E*). In *A* and *C*, ordinate denotes the reciprocal time constant (τ^{-1} in s^{-1}) at given potentials (abscissa in mV). ● denote the mean value for the τ^{-1} ($n = 5$ for *A* and $n = 4$ for *C*). Each straight line denotes the best fit for the data points (least-squares method). The slope factor was 12·6 mV for *A* and 13·1 mV for *C*. *B*, sample recordings plotted in *A*. The cell was hyperpolarized from the holding potential (V_h) of −64 to −95 mV for 1 s and then depolarized to −30 mV for 400 mV and finally clamped at five different potentials indicated (see inset for the protocol). Tail currents are superimposed on the baseline. *D*, sample recording plotted in *C*. The inward tail currents at −40, −60 and −80 mV are superimposed on the baseline. V_h was −50 mV. Pre-pulse was to −95 mV for 1 s. I_K was activated at −20 mV for 150 ms. *E*, deactivating inward tail current following the activated I_K at three different voltages indicated. Pre-pulse from V_h −60 to −110 mV for 1 s was followed by step depolarizations for 200 ms to activate I_K (see inset for the protocol). The polarity of the activated I_K was outward at −30 mV and inward at −50 mV (not illustrated here).

Steady-state inactivation

As described previously with regard to the results shown in Fig. 4*B*, the hyperpolarization-induced extra I_K reached a non-zero steady-state value instead of declining to zero. These observations (and results in Fig. 4*D* also), led us to hypothesize that the inactivation of I_K may be incomplete and that a fraction of I_K

remains to contribute to the steady-state membrane conductance. Therefore, the steady-state inactivation curve was studied for I_K.

The usual experimental protocol adopted for studying the steady-state inactivation curve of I_K is illustrated in Fig. 6*A*: the cell membrane potential was

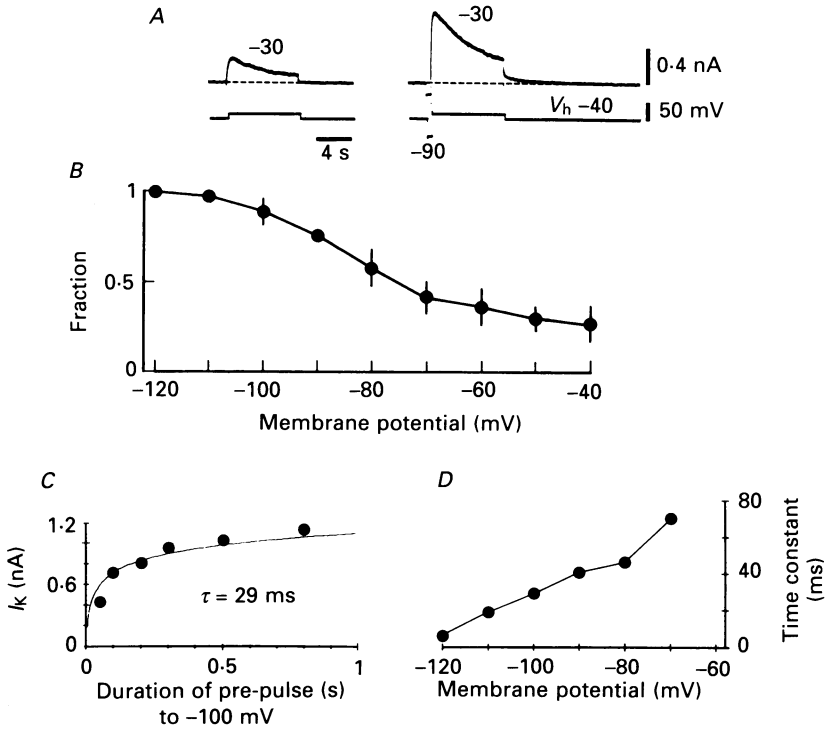

Fig. 6. Steady-state inactivation curve for I_K. *A*, I_K at −30 mV evoked by step depolarizations from −40 mV (left) and −90 mV (right). Results obtained from experiments of this type are summarized as the steady-state inactivation curve shown in *B*. ● and associated bars denote means ± and S.E.M. ($n = 5$) for the fractional I_K. *C*, the cell was held at −50 mV and subjected to 50 mV hyperpolarizing pre-pulses followed by step depolarizations to −20 mV. The peak amplitude of I_K at −20 mV is plotted (ordinate in nA) as a function of the duration of the pre-pulses (abscissa in s). ● denote the measured data and the continuous line denotes the best fit (least-squares method) having the time constant of 29 ms. *D*, relation between the time constant (ordinate in ms) for the removal of the inactivation and the level of the pre-pulses (abscissa in mV).

clamped at −40 mV and briefly (500–800 ms) commanded to hyperpolarized levels (range, −35 to −140 mV) before stepping to −30 mV. The peak amplitude of an outward current at −30 mV (*I*) was normalized with respect to its maximum amplitude (I_{max}) and then the I/I_{max} or the fractional I_K was plotted as a function of the membrane potential from which the cells were depolarized. The I_{max} was obtained with step commands from about −120 mV indicating the top of the steady-state I_K inactivation curve. The fractional I_K was not decreased to zero even at −40 mV in all five cells tested (Fig. 6*B*); about 30 mV less negative than the bottom of the steady-state activation curve of I_K shown in Fig. 1*B*. This was suggestive of

the presence of a steady-state 'window' current at 'subthreshold' potentials; in the normal Ringer solution, the resting membrane potential ranged from −65 to −78 mV and the 'threshold' for the generation of the action potential was about −40 mV.

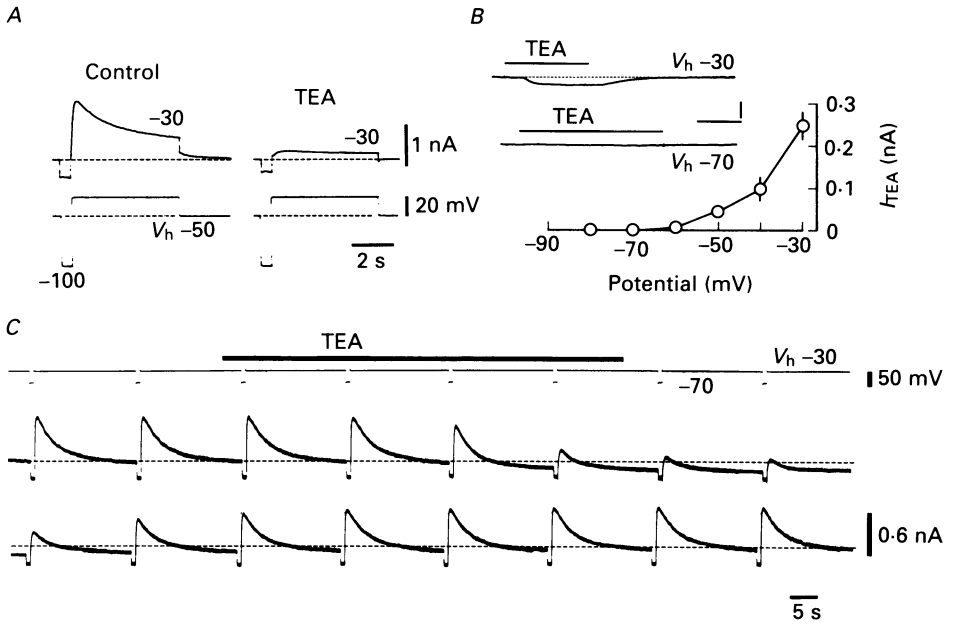

Fig. 7. Sensitivity of I_K to TEA. *A*, I_K was evoked by subjecting the cell to step hyperpolarizations from the holding potential (V_h) of −50 to −100 mV for 500 ms and then to step depolarizations to −30 mV for 5·5 s. TEA (30 mM) reduced the amplitude of I_K to about 8%. *B*, ordinate denotes the amplitude of an inward shift of the holding current caused by bath application of 20 mM-TEA for 3 min (indicated by I_{TEA}). Abscissa denotes V_h at which the amplitude of the I_{TEA} was measured. ○ and bars represent means ± S.E.M. ($n = 5$). Inset shows sample recordings plotted in the graph which were obtained from a single cell at a V_h of −30 to −70 mV. TEA (20 mM) was added to the superfusate for a period indicated by filled bars. Scales denote 0·5 nA and 1 min. *C*, the cell was clamped at a V_h of −30 mV and hyperpolarized to −70 mV for 800 ms (see the voltage trace shown at the top). I_K was evoked on repolarization from −70 to −30 mV (the current trace at the middle was followed by that at the bottom). TEA (20 mM) was added to the superfusate for a period (approximately 75 s) indicated by a filled bar. Two dashed lines denote the holding current level at −30 mV before the TEA application.

The time course with which the steady-state inactivation was removed could be examined by changing the duration of hyperpolarizing pre-pulses to fixed voltage (range, −70 to −120 mV). The peak amplitude of an outward current during step depolarizations to −30 mV was plotted as a function of the duration of the pre-pulses (range, 10–800 ms). At any given pre-pulse voltage, the steady-state inactivation of I_K was removed along with a single exponential time course (Fig. 6*C*) and the time constant was decreased from about 70 ms at −70 mV to about 10 ms at −120 mV (Fig. 6*D*). Quantitatively similar observations were made from two other cells.

Pharmacological profiles of I_K

Tetraethylammonium

Sensitivity of I_K to extracellularly applied TEA (3–50 mM) was examined by substituting sodium chloride in the standard solution with equimolar TEA chloride

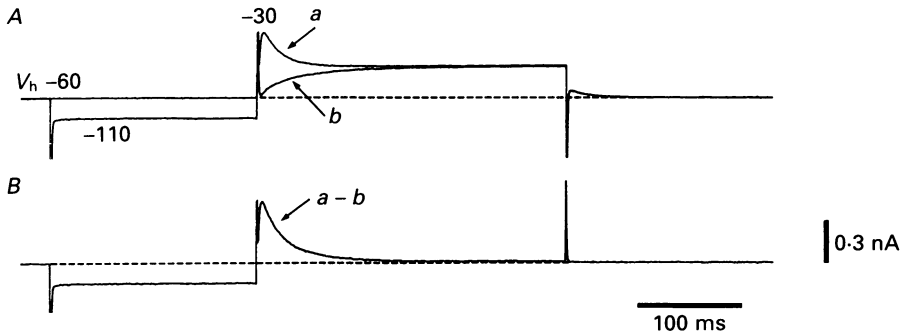

Fig. 8. Rapidly inactivating A-current. Results were obtained from a single cell superfused with a nominally calcium-free Ringer solution containing 30 mM-TEA (see Methods for its composition). The M-current was activated during 300 ms step depolarizations from the holding potential (V_h) of −60 to −30 mV. The M-current was superimposed by the A-current when the depolarizing step commands were preceded by hyperpolarizing pre-pulses to −110 mV for 200 ms. *A*, the M-current (current trace indicated by '*b*') and a mixture of the M- and A-current (current trace indicated by '*a*') are superimposed on the baseline. *B*, the A-current component is shown, which was given as a difference between the membrane currents with and without the pre-pulse (indicated by '*a*−*b*'). The A-current inactivated single exponentially with a time constant of $\tau = 30$ ms.

and in each experiment the effects of the equimolar substitution of sodium chloride with choline chloride were tested as a negative control. TEA (3–50 mM) decreased the peak amplitude of I_K in a concentration-dependent manner (Fig. 7). More than 90% inhibition of the current was obtained with 30 mM-TEA ($n = 4$) ($\sim$ 75% inhibition with 20 mM; $n = 5$).

In all five cells tested, TEA (10–50 mM) caused a concentration-dependent inward shift of the holding current at −30 mV (Fig. 7*B*), and at the same time, the I_K amplitude was reduced when the current was evoked at the termination of brief (0·5–1 s) step hyperpolarizations to −70 mV (Fig. 7*C*). The TEA-induced inward shift of the holding current progressively became of smaller amplitude as the holding potential was made more negative than −30 mV and simply disappeared when the cells were clamped at potentials more negative than the bottom of the steady-state activation curve (Fig. 7*B*). These observations were consistent with a flat envelope of the clamping currents at −70 mV in Fig. 7*C*.

In a nominally calcium-free and TEA-rich (30 mM) Ringer solution which did not contain 4-AP (see Methods), step depolarizations from the holding potentials less negative than −60 to −30 mV evoked the M-current (Fig. 8*A*). The M-current was superimposed by the A-current when the depolarizations were preceded by hyperpolarizing pre-pulses to potentials negative to −70 mV (Fig. 8*A*). Subtraction

of the clamping currents without the pre-pulses from those which have been recorded with the pre-pulses gave a measurement of the inactivation time constant of the A-current which is not contaminated by the M-current (Fig. 8*B*). Experiments of this type have shown that the A-current inactivated single exponentially with time ($\tau \simeq 30$ ms at -30 mV, $n = 4$).

Apamin and β-bungarotoxin

I_K was not significantly affected by apamin (10–100 nM) or β-bungarotoxin (30 nM) ($n = 3$ for each toxin).

Forskolin and related drugs

In all ten cells tested, forskolin (10 μM) reduced the peak amplitude of I_K to about 65% (to about 25% with 30 μM) (Fig. 9*A*). The minimum effective concentration of the drug exerting detectable reduction in the I_K amplitude ranged from 0·3 to 1 μM. The control I_K was regained in about 5 min when the bath application of the drug was discontinued. 1,9-Dideoxy forskolin (1–10 μM) mimicked the forskolin actions on I_K (Fig. 9*B*) implying that the forskolin action on I_K is unrelated to the forskolin-induced stimulation of adenylate cyclase (Seamon & Daly, 1986). Compatible with such a speculation, this forskolin analogue (10 μM) failed to enhance the H-current ($n = 5$, Fig. 9*D*) (see Tokimasa & Akasu, 1990*a*, *b*). A hydrophilic forskolin analogue, 7-*O*-hemisuccinyl-7-deacetyl forskolin (succinyl forskolin, 10 μM) reduced the I_K amplitude only to 82% in one cell but did not significantly affect I_K in two other cells (Fig. 9*C*).

Bath application of membrane-permeable cyclic AMP analogues such as 8-bromoadenosine 3′,5′-cyclic monophosphate (8-bromo-cyclic AMP, 0·1–1 mM) and dibutyryl cyclic AMP (1 mM) enhanced the H-current ($n = 4$ for each analogue) but did not have any significant effect on I_K ($n = 4$ for each analogue). A phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 0·1–1 mM), mimicked the forskolin actions on the H-current ($n = 6$) but not on I_K ($n = 4$). I_K was not significantly affected by a protein kinase inhibitor H-8 (10 μM) and a protein kinase activator phorbol 12-myristate 13-acetate (PMA, 1–3 μM) ($n = 2$ and 3 for each drug respectively).

In the normal Ringer solution, bath application of forskolin (10 μM) caused a membrane depolarization in two unclamped cells, the peak amplitude of which was 3 and 7 mV at the resting potential of -72 and -79 mV, respectively. The depolarization was associated with a decreased input resistance. When these cells were depolarized to -50 mV, the peak amplitude of the forskolin-induced depolarization was 4 and 5 mV and the depolarization was now associated with an increased input resistance. 8-Bromo-cyclic AMP (1 mM) also caused a membrane depolarization but only at or near rest and this was associated with decreased input resistance. 1,9-Dideoxy forskolin (10 μM) also caused a depolarization but only at depolarized potentials with respect to rest and this was associated with an increased input resistance. These observations implied that forskolin causes a membrane depolarization by enhancing the depolarizing influence of the H-current (see Tokimasa & Akasu, 1990*a*) and inhibiting the hyperpolarizing influence of I_K.

Delayed potassium current in sympathetic neurones

The purpose of the experiments described in this section was to demonstrate a slowly inactivating 'delayed' potassium current (I_K) in cultured bull-frog sympathetic B-neurones (Adams *et al.* 1982). In all experiments, TTX (3 μM), 4-AP

(1 mM) and caesium (1–2 mM) were added to the superfusate to eliminate the inward sodium current, the A-current and the H-current (Adams *et al.* 1982; Jones, 1987; Tokimasa & Akasu, 1990*a*, *c*).

Figure 10*A* illustrates the usual experimental protocol which has been adopted for studying the M-current (Adams *et al.* 1982): the cells were clamped at −40 mV and

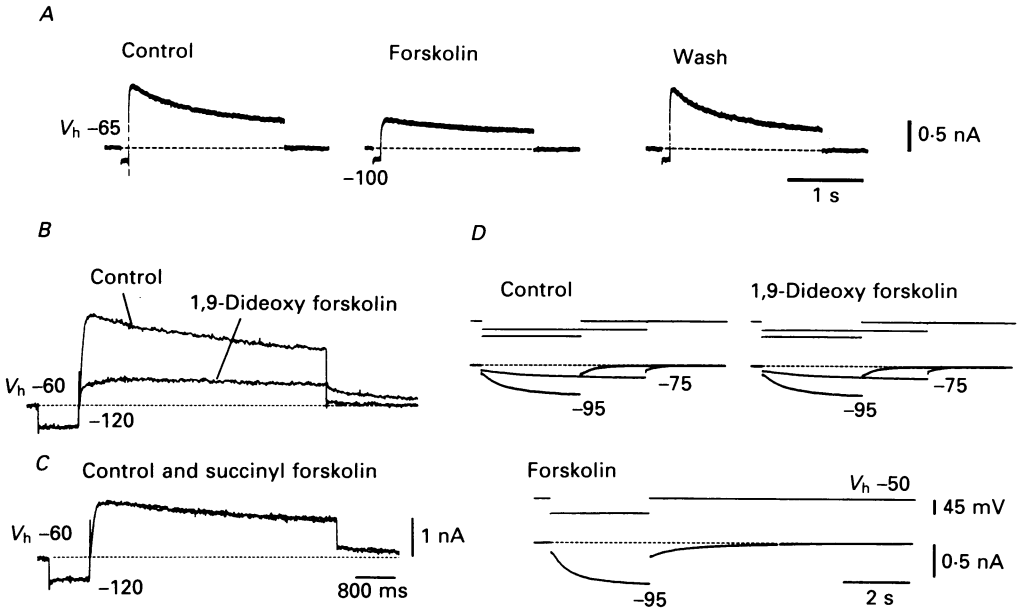

Fig. 9. I_K inhibition by forskolin and its analogue. Results in *A–D* were obtained from four different cells. *A*, the cell was hyperpolarized from the holding potential (V_h) of −65 to −100 mV and then depolarized to −30 mV before stepping back to −65 mV. Traces from the left to right are I_K in control (left), in the presence of 30 μM-forskolin (middle) and after wash-out with the drug-free solution (right). *B* and *C*, the cells were hyperpolarized from a V_h of −60 to −120 mV for 800 ms and then depolarized to −30 mV before stepping back to −60 mV. 1,9-Dideoxy forskolin (10 μM) inhibited I_K (*B*) while succinyl forskolin (10 μM) was ineffective on I_K (*C*). Calibrations in *C* are also applicable for records in *B*. *D*, the H-current (I_H) was activated during step hyperpolarizations to −75 and −95 mV from a V_h of −50 mV. 1,9-Dideoxy forskolin (10 μM) failed to enhance I_H while forskolin (3 μM) clearly increased the amplitude of I_H.

subjected to hyperpolarizing step commands (5–70 mV, 300–800 ms). The deactivating M-current during the commands produced relaxations on the current trace according to the voltage dependence of its deactivating rate constant (Adams *et al.* 1982). Upon stepping back to the holding potential of −40 mV from potentials more negative than −60 mV, the reactivating M-current was masked by a slowly inactivating I_K of which amplitude progressively became larger as the amplitude of the hyperpolarizing step commands was increased (Fig. 10*A*).

The cells were clamped at −40 mV and hyperpolarized to −90 mV for 500 ms and then polarized to various potential levels for 8·5 s before stepping back to −40 mV (Fig. 10*B*). The polarity of a mixture of the M-current and I_K was outward at

-50 mV with $[K^+]_o$ in the superfusate at 2 mM (Fig. 10*B*). The polarity of the current was outward -30 mV but inward at -40 mV when $[K^+]_o$ was elevated to 20 mM (Fig. 10*B*). This indicated that the reversal potential of I_K lies between -30 and -40 mV in a potassium-rich (20 mM) solution; under these conditions, the

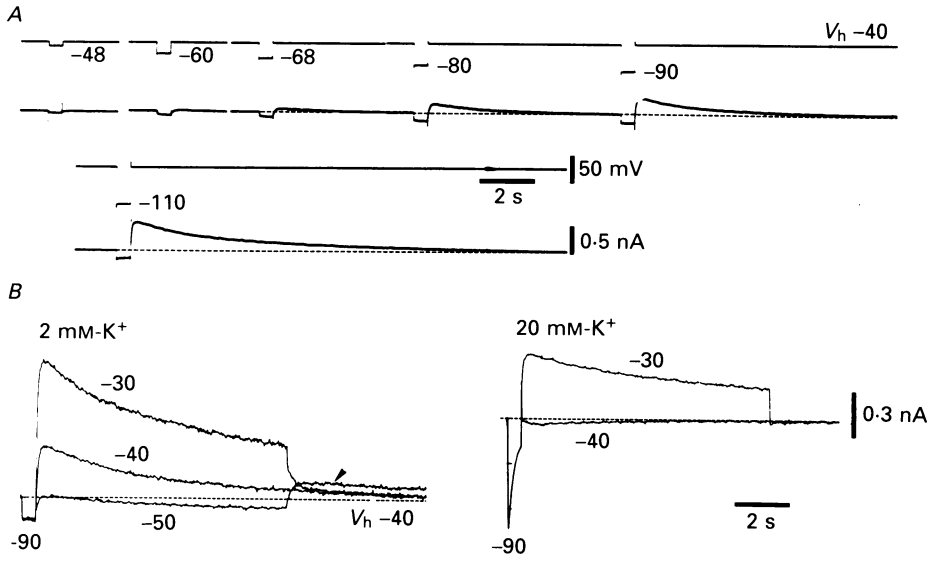

Fig. 10. A slowly inactivating I_K in sympathetic neurones. The cell was superfused with Ringer solution containing TTX (3 μM) and caesium (1 mM). The holding potential (V_h) was -40 mV. *A*, a slowly inactivating I_K elicited at the termination of 500 ms step hyperpolarizations to -60, -68, -80, -90 and -110 mV. *B*, step hyperpolarizations to -90 mV for 1 s were used as the hyperpolarizing pre-pulses for the subsequent step depolarizations to -50, -40 and -30 mV (left, $[K^+]_o = 2$ mM) and to -40 and -30 mV (right, $[K^+]_o = 20$ mM).

reversal potential of the M-current lies between -30 and -40 mV (Adams *et al.* 1982; Tokimasa & Akasu, 1990*c*). Therefore, it was confirmed that I_K is a potassium current. The inactivation of I_K was also incomplete at least during 8·5 s polarization at -50 mV since I_K could be evoked upon stepping back to -40 from -50 mV (indicated by an arrow in Fig. 10*B*).

I_K could be observed in a nominally calcium-free Ringer solution containing magnesium (4 mM), cobalt (1 mM), nickel (1 mM), barium (2 mM), caesium (1 mM) and 4-AP (1 mM). Unlike the sensory I_K, however, the reciprocal time constant of the tail currents (τ^{-1} in s^{-1}) increased e-fold with a $28{\cdot}1 \pm 1{\cdot}7$ mV hyperpolarization in four cells (Adams *et al.* 1982).

DISCUSSION

The results in the present study have demonstrated a slowly inactivating 'delayed' potassium current in cultured bull-frog sensory and sympathetic neurones. The current was incompletely inactivated near the resting membrane potential and hence bath application of TEA caused a membrane depolarization.

Identity of I_K in primary afferent neurones

Five lines of evidence in the present study have suggested that a slowly inactivating potassium current which we called I_K could be classified as a delayed rectifier. First, I_K was not 'calcium activated'. Second, the time course with which I_K inactivates was about ten to twenty times slower than that of the A-current. Third, I_K activated with a sigmoidal time course. Fourth, I_K deactivated single exponentially with time. Finally, I_K was sensitive to TEA. Insensitivity of I_K to PMA, an activator of protein kinase C (Nishizuka, 1984), implied that the intracellular control mechanisms for I_K are distinct from those which have been proposed for the M-current (Brown, Marrion & Smart, 1989; Tokimasa & Akasu, 1990*b*).

It seems likely that I_K is distinct from two other inactivating potassium currents known to occur in mammalian sensory neurones. One is a TEA-insensitive, slowly inactivating current of rat nodose ganglion cells (Stansfeld *et al.* 1986) and the other is a fast transient current ($I_{K(f)}$) of guinea-pig dorsal root ganglion cells (Penner, Petersen, Pierau & Dreyer, 1986; Petersen, Penner, Pierau & Dreyer, 1986). However, it might be possible that I_K corresponds to a slower component to an ensemble average current of a 20 pS channel of guinea-pig dorsal root ganglion cells since the current is insensitive to 4-AP (5 mM) applied in the outside-out configuration and the slower component inactivates with the time constant of about 4 s (Kasai *et al.* 1986).

Previous study on sympathetic neurones

A previous study on bull-frog sympathetic neurones has described the kinetic scheme for the activation, deactivation and inactivation of the delayed rectifier present in a nominally calcium-free and potassium-rich (25 or 47 mM) Ringer solution containing TTX (0·5 μM) and cadmium (0·2–0·5 mM) (Adams *et al.* 1982). In that study, the steady-state conductance–voltage relation had a threshold near -25 mV when it was observed (Fig. 14*F* in Adams *et al.* 1982) as opposed to -70 mV when it was predicted from the kinetic scheme based on the reciprocal time constants observed for the on and off currents (Fig. 16 in Adams *et al.* 1982). Such a discrepancy has not yet been discussed systemically in subsequent whole-cell patch-clamp experiments (Goh, Kelly & Pennefather, 1989; Jones, 1989; Tokimasa & Akasu, 1990*c*). It has already been shown that the sympathetic delayed rectifier does show a slow inactivation (Fig. 18 in Adams *et al.* 1982) and therefore previous studies on dissociated neurones by ourselves and those by others (see above) may have studied insufficiently the mechanisms underlying the steady-state inactivation of the delayed rectifier. In this context, recent observations by Pfaffinger (1988) are of some interest. He noticed an outward current (I_{KP}) which is activated at -35 mV but which can be activated after removing inactivation by pre-pulsing the voltage to potentials negative to -70 mV; once activated, I_{KP} inactivates with a time constant of about 5 s at -35 mV. Because of this characteristic behaviour, it might be possible that I_{KP} is in fact identical or at least similar to I_K described in the present study.

Alternatively, it is possible to speculate that there are 'low threshold' and 'high

threshold' delayed rectifiers and the former current could be detected only when the hyperpolarizing pre-pulses are used. However, it should be noticed that there is not any significant difference between the presumed currents in terms of their sensitivity to TEA ($\geqslant 90\%$ block with 30 mM) and the voltage dependence of the reciprocal time constant for the deactivation (e-fold increase per $\simeq 30$ mV) (Adams *et al.* 1982; Tokimasa & Akasu, 1990*c*; the present study).

Since the inactivation of I_K was incomplete at the potential range over which the M-current is present, I_K could possibly be significant physiologically in controlling the membrane excitability in bull-frog sympathetic neurones particularly at slightly depolarized potentials with respect to rest. Consistent with this, it has been shown that TEA (4 mM) clearly reduces the slope conductance in the steady-state I–V curve at potentials less negative than -60 mV (Jones, 1989).

It seems that a slowly inactivating, 4-AP insensitive outward current of guinea-pig inferior mesenteric ganglion cells (Cassell, Clark & McLachlan, 1986) might correspond to I_K of bull-frog sympathetic neurones. Our preliminary experiments using a single-microelectrode voltage clamp have shown that an I_K-like potassium current also occurs in rabbit bladder parasympathetic ganglion cells (T. Nishimura, T. Tokimasa & T. Akasu, unpublished data).

Slowly inactivating potassium current in brain

Recent studies in mammalian hippocampus pyramidal cells have demonstrated a delayed potassium current (D-current; Storm, 1988) showing much higher sensitivity to 4-AP than the A-current (Gustafsson, Galvan, Grafe & Wigström, 1982) and a delayed rectifier (K-current; Segal & Barker, 1984; Numann, Wadman & Wong, 1987). It is possible that I_K may correspond to a component of the K-current of Numann *et al.* (1987) present in a cobalt-containing solution.

More recently, a slowly inactivating potassium current was found in rat hypothalamus histaminergic neurones (Greene, Haas & Reiner, 1990): the current has been termed a slow A-current in spite of its insensitivity to 4-AP. It is less likely that the slow A-current corresponds to I_K since the slow A-current is completely inactivated at potentials positive to -70 mV and the current is insensitive to TEA.

The primary structure of voltage-dependent potassium channels is now being elucidated by molecular cloning, and it is already clear that many different channels can be distinguished (Timpe, Schwarz, Tempel, Papazian, Jan & Jan, 1988; Christie, Adelman, Douglass & North, 1989; Frech, VanDongen, Schuster, Brown & Joho, 1989; Stühmer, Ruppersberg, Schröter, Sakmann, Stocker, Giese, Perschke, Baumann & Pongs, 1989). Although these channels have different properties when they are expressed from cDNA clones, some of which resemble the 'delayed rectifier' and some resemble the 'transient' current (see Pongs, 1989), it is far from clear how these channels correspond to those observed in vertebrate neurones. Indeed, the possibility of channel formation by polymerization of distinct subunits (hetero-polymerization: Christie, North, Osborne, Douglass & Adelman, 1990) may make this information very difficult to obtain.

Forskolin actions

Three lines of evidence in the present study have suggested that the forskolin-induced I_K inhibition is unconnected with phosphorylation of ion channels by cyclic AMP-dependent protein kinase (Nestler & Greengard, 1984; Kaczmarek & Levitan, 1987). First, 1,9-dideoxy forskolin, which does not stimulate adenylate cyclase (Seamon & Daly, 1981, 1986), mimicked the forskolin actions on I_K but not on the H-current (see Tokimasa & Akasu, 1990*a*, *b*). Second, 8-bromo-cyclic AMP, dibutyryl cyclic AMP and IBMX mimicked the forskolin actions on the H-current but not on I_K. Finally, a protein kinase inhibitor H-8 (Hidaka, Inagaki, Kawamoto & Sasaki, 1984) which inhibits the H-current (Tokimasa & Akasu, 1990*a*), failed to affect I_K. These observations rather suggest that forskolin directly alters gating of potassium channels involved such as that occurring in some invertebrate nerve cells and clonal tumour cells (Coombs & Thompson, 1987; Watanabe & Gola, 1987; Hoshi, Garber & Aldrich, 1988; Harris-Warrick, 1989). It seems that a lipid-soluble form of forskolin is necessary to affect gating of the potassium channels since a hydrophilic forskolin analogue (succinyl forskolin) negligibly reduces the I_K amplitude. However, it should be noted that the wash-out of the forskolin actions on I_K was significantly faster ($\leqslant$ 5 min, present study) than that for the H-current (30–40 min, Tokimasa & Akasu, 1990*a*).

Functions of I_K

The results in the present study have demonstrated a TEA-sensitive 'window' current near the resting potential which resulted from an incomplete inactivation of I_K indicating a persistent outward current near the resting membrane potential. Co-activation of the current with the M-current in sympathetic neurones led us to speculate that there might be some neurotransmitters which preferentially modulate I_K without significantly affecting the M-current and *vice versa*.

In conclusion, the present study has demonstrated a TEA-sensitive delayed rectifier type potassium current which inactivates much more slowly than the A-current. The current did not require an inward calcium current for its activation and the current constituted, together with the M-current, a persistent outward current at or near the resting potential.

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